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Physicochemical and Metabolic Properties of Modified Metallothioneins

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Attempts to crosslink metallothionein through lysine residues have resulted in trapping of octameric structures. Two such polymers have been characterized. They are of a critical size for glomerular filtration, and unique tissue distributions of cadmium arise when they are injected into rats.

The biocomplexes of cadmium may play an important role in the tissue deposition and toxicity of this metal (*1*). Injection of Cd^{2+} salts into experimental animals results initially in major deposition of Cd in the liver, whereas injected Cd-metallothionein (Cd–MT) is accumulated mainly in the proximal renal tubular cells. Various organic chelates of Cd show an intermediate pattern of distribution (*2*). In order better to understand the factors controlling the binding of metals to MT, and the effects of these factors on Cd deposition and toxicity, we are investigating chemically modified MTs as novel chelators of Cd. Here we report the results obtained with two crosslinked MT polymers.

Two distinct MT polymers have been prepared by reaction of Cd-induced rat liver MT–II with bifunctional crosslinking reagents (Fig. 1). Controlled polymerization with glutaraldehyde followed by $NaBH_4$ reduction of the resulting Schiff base produces a polymer with a molecular weight of 58,900 daltons, designated GA–MT. A high yield of this one polymer is demonstrated by gel electrophoresis. Reaction of MT with dimethylsuberimidate similarly produces a single polymer (DMS–MT), with a molecular weight of 49,000 daltons, in greater than 80% yield. This represents an octamer of MT. The paucity of lower and higher order polymers, and the similar pattern of polymerization in both reaction mixtures, indicate trapping of a pre-associated octamer of MT, rather than isolation of thermodynamic reaction products. The higher molecular weight of GA–MT may then be accounted for by a greater number of monofunctionalized lysine residues (when bis-lysine is determined by amino acid analysis of appropriately prepared standards; see Table 1), and a tendency of this reagent to form glutaraldehyde polymers linked to protein. For subsequent studies, the polymers were purified by Sephadex G–75 fractionation.

The physical characteristics of these polymers are summarized in Table 1. The Stokes' radii determined from a calibrated Sephadex G–100 column have been used to calculate frictional ratios, f/f_o. A ratio of 1.3 for MT–II, indicative of the known asymmetry of this molecule, decreased to near unity for both polymers, demonstrating the globular nature of the octameric cluster. Sephadex G–75 fractionations and atomic absorption spectrophotometry demonstrate loss of 1 g atom of Cd per monomeric subunit from GA–MT, but the metal content of DMS–MT is identical to that of monomer (MT–II). Concomitant with loss of at least one binding site, the pI of GA–MT rises to 5.2 from the value of 4.6 found for MT–II and DMS–MT. Amino acid analysis and *p*–hydroxymercuribenzoate titrations show that the thiol content of each polymer is preserved, and any modifications are of a nonoxidizing nature and confined to lysine residues.

The tissue distributions of Cd from polymers were compared with that of Cd–MT and $CdCl_2$ in rats. Male Sprague-Dawley rats (150–200 g) were injected intravenously with low doses of Cd (7 ± 1 μg/kg) labeled with ^{109}Cd, as either $CdCl_2$, Cd–MT–II, Cd–GA–MT, or Cd–DMS–MT, and sacrificed at 3 or 24 hr. Differences in distribution of ^{109}Cd are shown in Figure 2. At 3 hr following

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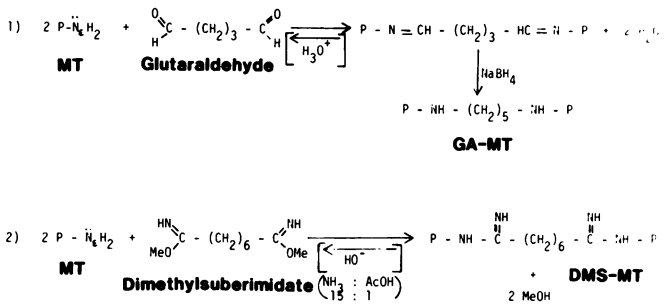

FIGURE 1. Reaction schemes for preparation of MT polymers. The reactions show the polymerization of MT through ε-amino groups of lysine residues, using glutaraldehyde (reaction 1) or dimethyl suberimidate (reaction 2).

$CdCl_2$ administration, Cd is found primarily in liver, while Cd–MT injection results in most of the dose of Cd being deposited in kidney. Both forms of Cd have been effectively cleared from circulation. The injection of polymers results in an intermediate Cd distribution with respect to liver and kidney. About 25% of the Cd given as DMS–MT is found in kidney, while a greater amount of the metal of GA–MT is recovered from liver. Furthermore, significant amounts of both polymers remain in circulation at 3 hr. This is especially true of GA–MT, which appears not to be as readily cleared by renal filtration. Significantly higher amounts of Cd are found in spleen when either polymer is administered, than when $CdCl_2$ or MT–II are given. The results are similar to a previous publication from our laboratory on the metabolism of a MT polymer in mice (*3*). This may in part account for the enhanced antigenicity reported for a polymerized MT (*4*) and probably reflects the prolonged circulation time of these species. There are no significant differences in the relative tissue levels of Cd between 3 and 24 hr for any form of administered Cd, with the exception of removal of polymer from plasma at the longer time period. Initial studies indicate that the reticuloendothelial system, particularly Kupffer cells in liver, plays a part in clearing these polymers from plasma.

It is interesting to note that although the Stokes' radii of GA–MT and DMS–MT are quite similar, they fall in a critical region for molecular sieving in the glomerulus, as determined by Pesce et al. (*5*). If the sieving coefficients of each of

Table 1. Summary of physicochemical properties of MT and its polymers.

	MT-II	GA-MT-II	DMS-MT-II
MW[a]	6,250	58,900	49,000
MW[b]	6,200	—	—
f/f_0	1.3	1.06	1.04
Stokes' radius, Å	16.0	27.6	25.4
pI[c]	4.6	5.2	4.6
Lys[b]	9.0	2.0	5.0
Bis-Lys[b]	—	3.0	2.0
Cys[b,d]	17	16	17
– SH[e]	18	18	18
– SH: (Cd + Zn)[f]	3.0	4.3	3.0
Cd/protein[g]	5.2	4.2	5.2
Zn/protein[g]	0.8	0	0.8
Cu/protein[g]	<0.2	—	—

[a]Determined by denaturing gel electrophoresis of S-carboxymethyl derivatives.
[b]By amino acid analysis.
[c]By isoelectric focusing of pH 4–6 gradients.
[d]Following performic acid oxidation.
[e]By *p*-hydroxymercuribenzoate (PMB) titration.
[f]Based on PMB titration and atomic absorption; mole ratio.
[g]Mole ratio, by atomic absorption spectrophotometry.

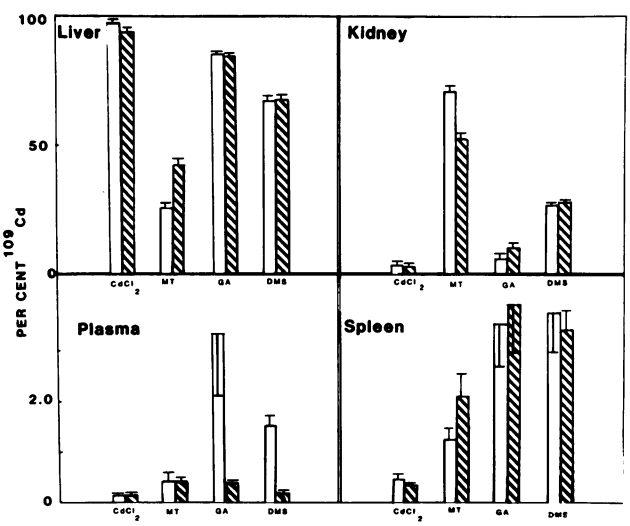

FIGURE 2. Tissue distribution of various forms of Cd. Bars show the percent of the recovered ^{109}Cd (each is the mean of five animals ± SD) found in the indicated tissues, based on the total ^{109}Cd recovered from liver, kidney, spleen, pancreas, duodenum and plasma. In all cases total recoveries were > 85% of administered dose. Rats were injected intravenously with ^{109}Cd-labeled $CdCl_2$, Cd-MT-II, Cd-GA-MT, or Cd-DMS-MT, and sacrificed at 3 or 24 hr. ^{109}Cd was determined by counting of γ-radiation.

the three MT species investigated here are calculated assuming direct proportionality to the measured renal uptake, then our observed relationship of sieving coefficient to Stokes' radius is in excellent agreement with the data of Pesce et al. (*5*). If such octameric aggregates of MT do occur *in vivo,* their dimensions are expected to fall in a critical range for glomerular filtration.

Full details of this work will be published elsewhere (*6*).

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